In Japan, cytotoxicity tests for identifying non-irritating ingredients have been presented in *Guidance on Alternative Appraisal Methods for Determining the Eye Irritation Potential of Cosmetic Raw Materials* (5). This document was based on the results of a validation programme entitled *Study on Test Methods to Evaluate the Safety of Cosmetics Containing New Ingredients*, supported by funds from the Japanese Ministry of Health and Welfare (6). The guidance states that, if a test substance is found to be non-irritant on the basis of alternative methods alone, and it will not be formulated into products at a concentration in excess of 10%, then it may be appraised as a non-irritant without additional animal testing. The guidance also notes that artificial dermal models can be used for the identification and classification of non-irritants and irritants.

We re-analysed the results of the Japanese validation study, and designed a novel tier evaluation system, by combining SIRC monolayer cell cultures and a three-dimensional LDM (7). The cytotoxic endpoints were measured by means of Crystal Violet staining (in the SIRC-CVS assay) and MTT reduction (in the LDM-MTT assay), respectively. The former method was developed by Itagaki *et al.* (8) and the latter method by Bell *et al.* (9) and Gay *et al.* (10). A schematic illustration of the tier evaluation system proposed for the identification of non-irritating ingredients is shown in Figure 1. In this study, the effectiveness of this tier system was examined by assessing 59 cosmetic ingredients, for which *in vivo* data had previously been reported. The LDM-MTT assay was also applied to an additional 73 ingredients, for which MAS scores in the Draize eye test were available.

Materials and Methods

Test substances

The test substances used are shown in Table 1. The 59 substances were selected from among chemicals for which *in vivo* eye irritation data were already available (11–60). They were purchased from Alfa Aesar (Karlsruhe, Germany), Fluorochem Ltd (Old Glossop, Derbyshire, UK), MP Biomedicals (Irvine, CA, USA), Tokyo Chemical Industry Co. Ltd (TCI; Tokyo, Japan), and Wako Pure Chemical Industries, Ltd (Wako; Osaka, Japan). The other 73 substances were selected from among cosmetic ingredients for which MAS values in the Draize eye test were available (61).

SIRC (Statens Serum Institut Rabbit Cornea) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The MATREX™ kit was purchased from Toyobo Co. Ltd (Osaka, Japan). The kit contains a LDM, which consists of human dermal fibroblasts cultured in a bovine type-I collagen lattice, to maintain a three-dimensional structure. Crystal Violet and MTT were purchased from Wako Pure Chemical Industries, Ltd and Dojindo Laboratories (Kumamoto, Japan), respectively.

Procedure

The cytotoxicity testing with SIRC cell monolayer cultures was performed on the basis of the method reported by Tani *et al.* (62). The SIRC cells were grown in Eagle's Minimal Essential Medium (MEM; Nissui Pharmaceutical Co. Ltd, Tokyo, Japan), containing sodium bicarbonate and supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA), 1% (v/v) antibiotic– antimycotic (100× mixture; Invitrogen Corporation, Carlsbad, CA, USA) and 0.2M glutamine. The cultures were maintained in flasks at 37°C in a humidified incubator containing 5% (v/v) CO_2 in air, and routinely passaged on reaching 80–90% confluence. The cells were collected by trypsinisation, when required.

Aliquots of cell suspension (100µl; 3×10^4 cells/well) were gently introduced into wells into which test substances at various concentrations had been added beforehand. The stepwise determination of whether a test substance dissolved, or whether it was suspended uniformly, was based on visual observation. The solvents used for dilution were culture medium, phosphate-buffered saline without calcium and magnesium [PBS(–)], dimethyl sulphoxide (DMSO) or ethanol, and the appropriate solvent was selected on the basis of the solubility characteristics of the test substance. The maximal concentrations of PBS(–), DMSO and ethanol for application to the cells were 10, 1 and 1% (all v/v), respectively. The plates were left undisturbed for 20 minutes, to allow the cells to settle on the bottom of the wells. The test substances were incubated with the cells for 72 hours at 37°C in a humidified incubator containing 5% CO_2 in air. Dead cells were washed away with PBS(–), then cells attached to the bottom of the plate were fixed and stained with 0.4% (w/v) Crystal Violet solution in methanol for 30 minutes. Absorbance at 588nm was measured with an automatic microplate reader. The absorbance of control wells, which contained no test substance, was regarded as 100%, and the percentage absorbance for each well was calculated. The concentration at which the growth of cells was inhibited to 50% of the control (IC50) was obtained from the dose–response curve.

The LDM test was conducted according to a standard operating procedure (SOP) based on the kit supplier's protocol. The required number

Table 1: Characterisation of the fifty-nine test substances

No.	Substance	CAS No.	Supplier[a] (*in vitro* test)	*In vivo* data reported previously[b]			
				Classification at 10% conc.	Classification at the applied conc.	Corneal damage (C) or MAS (M)	Ref.
1	2-Bromo-2-nitropropane-1,3-diol	52-51-7	Fluorochem	Positive	Positive at 100, 20, 10, 5%, Negative at 2, 0.5%	C	11, 12
2	Benzalkonium chloride	8001-54-5	Wako	Positive	Positive at 2, 1, 0.5%, Negative at 0.1, 0.01%	C	13
3	Cetrimonium chloride	112-02-7	Wako	Positive	Positive at 2.5, 1.2, 0.5%, Negative at 0.1%	C	14
4	Chlorhexidine digluconate	18472-51-0	Wako	Positive	Positive at 20, 2%, Negative at 0.05%	M	15
5	Chlorophene	120-32-1	Wako	Positive	Positive at 100, 3%, Negative at 1, 0.3%	C	16
6	Dioctyl sodium sulphosuccinate	577-11-7	Alfa Aesar	Positive	Positive at 10%, Negative at 2, 0.5%	M	17
7	Lauramide DEA	120-40-1	Wako	Positive	Positive at 20, 10%	C	18
8	Phenethyl alcohol	60-12-8	Wako	Positive	Positive at 100, 15, 5%, Negative at 0.3%	C	19
9	Stearalkonium chloride	122-19-0	Wako	Positive	Positive at 25, 4, 2.5%, Negative at 0.5%	M	20
10	TEA-lauryl sulphate	139-96-8	Wako	Positive	Positive at 20, 10, 5, 2.5, 1.25%	M	21
11	Acetyl tributyl citrate	77-90-7	Wako	Negative	Negative at 100%	C	22
12	Benzophenone-1	131-56-6	Wako	Negative	Positive at 100%, Negative at 16, 8, 4%	M	23
13	Benzophenone-2	131-55-5	Wako	Negative	Positive at 100%, Negative at 16, 8, 4%	M	24
14	Butylene glycol	107-88-0	Wako	Negative	Negative at 100, 10%	M	25
15	Carnauba wax	8015-86-9	Wako	Negative	Negative at 50%	M	26
16	Cetyl alcohol	36653-82-4	Wako	Negative	Negative at 100%	C	27
17	Cetyl palmitate	540-10-3	Wako	Negative	Negative at 100%	C	28
18	Decyl oleate	3687-46-5	Wako	Negative	Negative at 100%	M	29
19	Diazolidinyl urea	78491-02-8	MP Biomedicals	Negative	Negative at 30%	C	30
20	Diethylhexyl adipate	103-23-1	Wako	Negative	Negative at 100%	M	31
21	Diisopropyl adipate	6938-94-9	Wako	Negative	Negative at 100%	M	31
22	Ethylhexyl palmitate	29806-73-3	Wako	Negative	Negative at 100%	C	28
23	Ethylhexyl stearate	22047-49-0	Wako	Negative	Negative at 100%	M	32
24	Glyceryl stearate	11099-07-3	Wako	Negative	Negative at 100%	C	33
25	Hexylene glycol	107-41-5	Wako	Negative	Positive at 100%, Negative at 25%	C	34
26	Isocetyl stearate	25339-09-7	Wako	Negative	Negative at 100%	C	32
27	Isopropyl myristate	110-27-0	TCI	Negative	Negative at 100%	C	35
28	Isopropyl palmitate	142-91-6	Wako	Negative	Negative at 100%	C	28
29	Oleyl alcohol	143-28-2	Wako	Negative	Negative at 100%	M	36
30	PEG-2 stearate	106-11-6	Wako	Negative	Negative at 100%	M	37

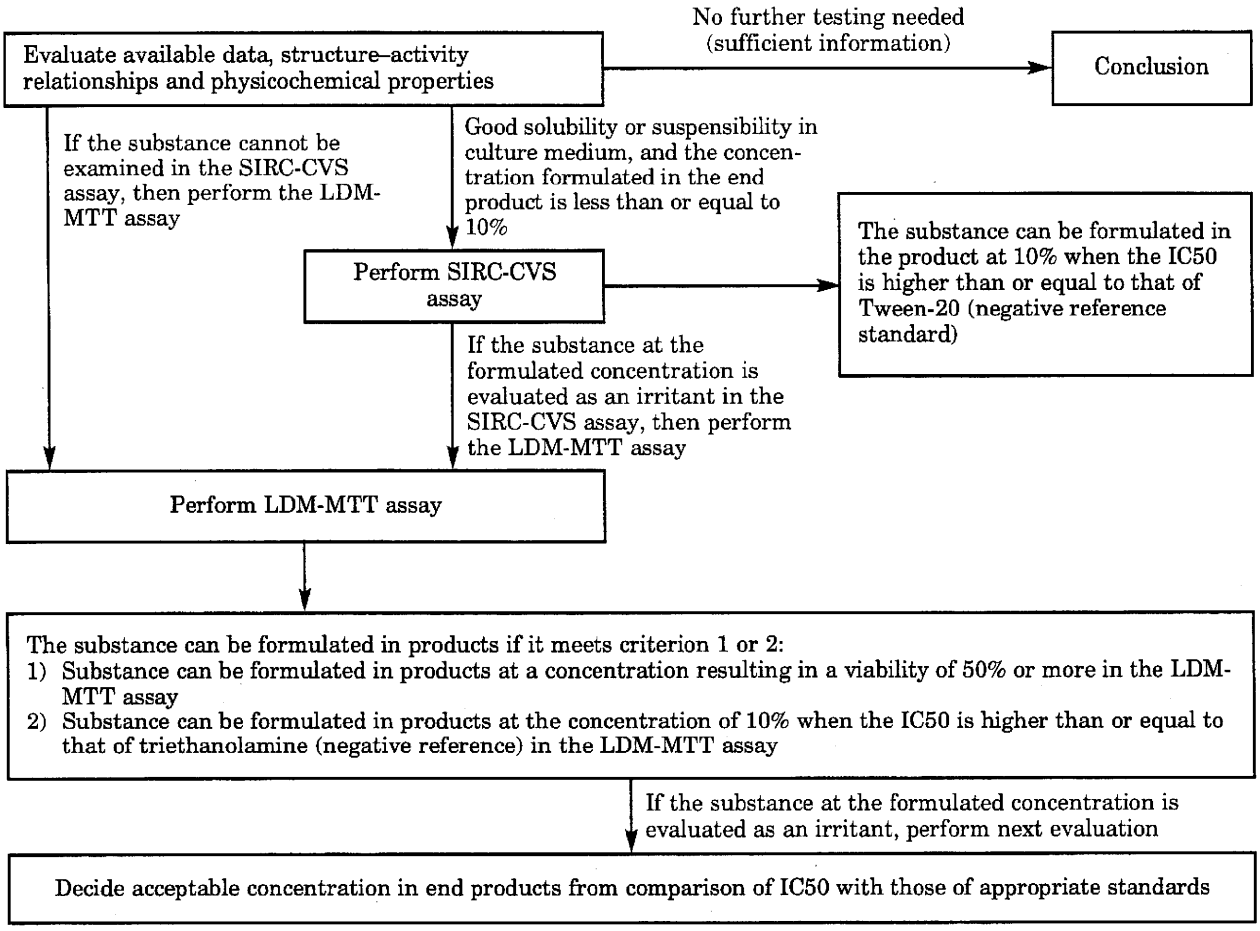
[a]Supplier means manufacturer of the material used in this study. [b]The in vivo *classification of positive or negative was based on the appearance or not of corneal damage (C) or an MAS value of 15 as a cut-off point (M), where reported MAS values are available. The classification was essentially based on whether or not corneal damage appeared after the application of 0.1ml of substance to the rabbit eye, without rinsing. However, where there were differences of test conditions, these were considered individually. For example, a case where corneal damage appeared after the application of 0.05ml was judged as positive. In cases without data at 10% concentration, the assessment of positive or negative at the concentration of 10% was made on the basis of dose–response analysis of each ingredient.*

Table 1: continued

No.	Substance	CAS No.	Supplier[a] (*in vitro* test)	*In vivo* data reported previously[b]			
				Classification at 10% conc.	Classification at the applied conc.	Corneal damage (C) or MAS (M)	Ref.
31	PEG-40 stearate	9004-99-3	Wako	Negative	Negative at 100%	M	37
32	Phytantriol	74563-64-7	Wako	Negative	Positive at 100, 23%, Negative at 10, 3%	C	38
33	Propylene carbonate	108-32-7	Wako	Negative	Negative at 100, 17.5, 10.5%	M	39
34	Castor seed oil	8001-79-4	Wako	Negative	Negative at 100%	C	40
35	Safflower oil	8001-23-8	Wako	Negative	Negative at 100%	M	41
36	Sesame (*Sesamum indicum*) oil	8008-74-0	Wako	Negative	Negative at 100%	M	42
37	Sodium dehydroacetate	4418-26-2	Wako	Negative	Negative at 100%	M	43
38	Sodium stearate	822-16-2	Wako	Negative	Negative at 100%	C	44
39	Sorbitan oleate	1338-43-8	Wako	Negative	Negative at 100%	C	45
40	Sorbitan sesquioleate	8007-43-0	Wako	Negative	Negative at 100, 30%	C	45
41	Sorbitan stearate	1338-41-6	Wako	Negative	Negative at 30%	C	45
42	Squalane	111-01-3	Wako	Negative	Negative at 100%	C	46
43	Steareth-2	9005-00-9	Wako	Negative	Negative at 60%	C	47
44	Steareth-20	9005-00-9	Wako	Negative	Negative at 60%	M	47
45	Stearyl alcohol	112-92-5	Wako	Negative	Negative at 100%	C	36
46	Triacetin	102-76-1	Wako	Negative	Negative at 100%	M	48
47	Triethylene glycol	112-27-6	Wako	Negative	Negative at 100%	C	49
48	Zinc stearate	557-05-1	Wako	Negative	Negative at 100%	C	44
49	Benzethonium chloride	121-54-0	TCI	Indistinguishable	Negative at 0.5%	C	50
50	Butoxyethanol	111-76-2	Wako	Indistinguishable	Positive at 100, 15%, Negative at 5%	C	51
51	Chloroxylenol	88-04-0	Wako	Indistinguishable	Positive at 100, 30%	M, C	52
52	Methoxyisopropyl acetate	108-65-6	Wako	Indistinguishable	Positive at 100%	C	53
53	Phenoxyethanol	122-99-6	Wako	Indistinguishable	Positive at 100%, Negative at 2.2%	C	54
54	Phenyl methyl pyrazolone	89-25-8	Wako	Indistinguishable	Negative at 0.66%	M	55
55	Resorcinol	108-46-3	Wako	Indistinguishable	Positive at 100%	C	56
56	Sodium hexametaphosphate	10124-56-8	Wako	Indistinguishable	Negative at 0.2%	C	57
57	Sodium lauroyl sarcosinate	137-16-6	Wako	Indistinguishable	Negative at 5%	C	58
58	Sodium naphthalenesulphonate	532-02-5	Wako	Indistinguishable	Positive at 100%, Negative at 2%	C	59
59	Triisopropanolamine	122-20-3	Wako	Indistinguishable	Positive at 100%	C	60

[a]Supplier means manufacturer of the material used in this study. [b]*The* in vivo *classification of positive or negative was based on the appearance or not of corneal damage (C) or an MAS value of 15 as a cut-off point (M), where reported MAS values are available. The classification was essentially based on whether or not corneal damage appeared after the application of 0.1ml of substance to the rabbit eye, without rinsing. However, where there were differences of test conditions, these were considered individually. For example, a case where corneal damage appeared after the application of 0.05ml was judged as positive. In cases without data at 10% concentration, the assessment of positive or negative at the concentration of 10% was made on the basis of dose–response analysis of each ingredient.*

Figure 1: A schematic illustration of a tier evaluation system featuring the SIRC-CVS assay and the LDM-MTT assay for the identification of non-irritating ingredients



of LDM samples were placed in wells of a six-well plate, and 5ml of assay medium were decanted onto the surface of each sample. The plate was left for 30 minutes at room temperature to remove any residual conditioned medium. Then the assay medium was aspirated from each well, and a 1.2ml aliquot of fresh assay medium was added underneath each LDM sample. A polyethylene ring was placed on the surface of the LDM sample, and silicone sealant was applied around the area of exposure. An 80µl (or 80mg, in the case of a solid) aliquot of test substance was then applied to the surface of the LDM, within the polyethylene ring. For discrimination between non-irritants and irritants at the concentration of 10%, the concentrations of the test samples used were usually 1, 5 and 10%, as according to the three-dose method of Hagino *et al.* (7). For prediction of the concentration at which an ingredient can be formulated into products without causing eye irritation, the concentration of the test samples was set at a similar concentration to that at which ingredients had been previously tested in *in vivo* tests. The solvent used for diluting the test substances was either distilled water, 50% (v/v) DMSO or ethylene glycol, selected on the basis of the solubility characteristics of the substance, as reported by Ohuchi *et al.* (63). When none of these solvents were suitable, liquid paraffin was employed (7). The stepwise determination of whether a test substance dissolved, or whether it was suspended uniformly, was based on visual observation. The LDM samples were exposed to the test substances for 24 hours at 37°C, in an incubator containing 5% CO_2 in air. After incubation, the test substances were removed from the wells by washing with the assay medium. Each treated LDM sample was submerged in 1.2ml of MTT solution (0.333mg/ml in assay medium) for 3–4 hours at 37°C. After exposure to the MTT solution, the centre of the LDM tissue was excised by using an 8mm diameter skin biopsy punch (RS-6330; Toyobo Co. Ltd, Osaka, Japan). As an indicator of cell viability, the MTT formazan dye was extracted with 0.3ml of iso-

propanol containing 0.04N HCl, over a 2-hour incubation period. The absorbance of the extract was measured at 570nm with a microplate reader (Benchmark Plus; Bio-Rad Laboratories, Hercules, CA, USA). Cell viability was expressed as the percentage of living cells relative to untreated controls or solvent-treated controls handled in the same manner, taken as the 100% value. The IC50 value was calculated by the interpolation of two dose–response data sets, if necessary.

Verification of the performance of the tier evaluation system

The performance of the tier evaluation system was measured in terms of its sensitivity, specificity, positive predictivity, negative predictivity and accuracy, as follows, where TP, TN, FP and FN are true-positive, true-negative, false-positive and false-negative, respectively:

Sensitivity =	TP/(TP + FN)
Specificity =	TN/(TN + FP)
Positive predictivity =	TP/(TP + FP)
Negative predictivity =	TN/(TN + FN)
Accuracy =	(TP + TN)/ (TP + TN + FP + FN)

Results

Prediction of eye irritation at a concentration of 10%

The tier evaluation system, comprising the SIRC-CVS assay and the LDM-MTT assay, was first assessed by applying it to the 59 selected substances, at a concentration of 10%.

The classifications in the SIRC-CVS assay and LDM-MTT assay were performed by using Tween-

Table 2: The predicted irritancy of 48 substances, based on SIRC-CVS assay results

		In vitro (classification by SIRC-CVS assay, with Tween-20 as a reference substance for non-irritancy)				
		+ ve		– ve		Could not be tested
		9		1		0
	+ve	2-Bromo-2-nitropropane-1,3-diol	(6.42 ± 0.85)	Phenethyl alcohol	(1830 ± 1360)	
		Benzalkonium chloride	(3.47 ± 0.47)			
		Cetrimonium chloride	(0.56 ± 0.16)			
		Chlorhexidine digluconate	(7.92 ± 3.92)			
		Chlorophene	(25.6 ± 9.1)			
		Dioctyl sodium sulphosuccinate	(81.3 ± 4.8)			
In vivo (classification by Draize eye test at 10% conc.)		Lauramide DEA	(18.3 ± 4.1)			
		Stearalkonium chloride	(2.66 ± 0.56)			
		TEA-lauryl sulphate	(117 ± 3)			
Corneal damage or MAS over 15 was classified as positive		10		17		11
	–ve	Benzophenone-1	(29.3 ± 8.0)	Butylene glycol	(>10,000)	Acetyl tributyl citrate
		Benzophenone-2	(53.4 ± 6.4)	Diethylhexyl adipate	(>1000)	Carnauba wax
		Cetyl alcohol	(25.1 ± 12.1)	Diisopropyl adipate	(633 ± 16)	Castor seed oil
		Diazolidinyl urea	(11.5 ± 7.7)	Ethylhexyl palmitate	(>10,000)	Cetyl palmitate
		Oleyl alcohol	(41.9 ± 13.3)	Hexylene glycol	(7500 ± 600)	Decyl oleate
		PEG-40 stearate	(230 ± 79)	Isocetyl stearate	(>1000)	Ethylhexyl stearate
		Phytantriol	(37.2 ± 11.8)	Isopropyl myristate	(>1000)	Glyceryl stearate
		Sodium stearate	(56.5 ± 8.2)	Isopropyl palmitate	(>1000)	PEG-2 stearate
		Steareth-2	(22.4 ± 5.4)	Propylene carbonate	(6050 ± 490)	Sorbitan stearate
		Steareth-20	(16.5 ± 8.3)	Safflower oil	(>1000)	Stearyl alcohol
				Sesame oil	(>1000)	Zinc stearate
				Sodium dehydroacetate	(860 ± 224)	
				Sorbitan oleate	(5170 ± 1560)	
				Sorbitan sesquioleate	(>10,000)	
				Squalane	(>1000)	
				Triacetin	(1780 ± 720)	
				Triethylene glycol	(>10,000)	

The results of the SIRC-CVS assay are shown as the average ± standard deviation (n = 3) of the IC50 value, in parentheses. The IC50 units are μg/μl. Tween-20 (IC50 = 501 ± 33μg/ml) was used as a reference substance for non-irritancy. The 11 substances that were insufficiently soluble to be tested are also shown in this table.

Table 3: Predicted irritancy in the LDM-MTT assay, of 19 substances positive in the SIRC-CVS assay and 11 substances with poor solubility in culture medium

		In vitro (classification by LDM-MTT assay, with triethanolamine as a reference substance for non-irritancy)	
		+ve	−ve
		9	0
In vivo (classification by Draize eye test at 10% conc.)	+ve	2-Bromo-2-nitropropane-1,3-diol Benzalkonium chloride Cetrimonium chloride Chlorhexidine digluconate Chlorophene Dioctyl sodium sulphosuccinate Lauramide DEA Stearalkonium chloride TEA-lauryl sulphate (<1 for above 9 substances)	
		7	14
Corneal damage or MAS over 15 was classified as positive	−ve	Benzophenone-1 (<1) Benzophenone-2 (<1) Diazolidinyl urea (<1) PEG-40 stearate (<1.3) Phytantriol (1.9) Sodium stearate (2.1) Steareth-20 (<1)	Acetyl tributyl citrate (100) Carnauba wax (100) Castor seed oil (100) Cetyl alcohol (>10) Cetyl palmitate (>10) Decyl oleate (100) Ethylhexyl stearate (100) Glyceryl stearate (100) Oleyl alcohol (100) PEG-2 stearate (100) Sorbitan stearate (100) Steareth-2 (>10) Stearyl alcohol (100) Zinc stearate (100)

The results of the LDM-MTT assay are presented, in parentheses, as the average IC50 value, expressed as a percentage (n = 2–3). Triethanolamine (IC50 = 4.6%) was used as a reference substance for non-irritancy.

20 and triethanolamine, respectively, as reference substances. It was confirmed that the results for the reference substances were similar to those obtained in the previous Japanese validation study, and that the *in vivo* test had been performed with the same reference substances as the *in vitro* test.

A reported *in vivo* eye irritation classification (positive or negative) at 10% concentration was available for 48 of the 59 ingredients. The classification of positive or negative had been performed essentially on the basis of whether or not corneal damage appeared after the application of 0.1ml of substance to the rabbit eye without rinsing, although there were individual differences of test conditions, and these were judged on a case-by-case basis. For example, corneal damage that appeared after the application of 0.05ml was classified as positive. If the MAS in the Draize eye test was reported in the paper, a value of 15 was used as the cut-off for positive or negative. In cases lacking data for a 10% concentration, the classification of positive or negative at this concentration was made on the basis of a dose–response analysis. The numbers of ingredients classified as positive and negative were 10 and 38, respectively, as shown in Table 1. When the classifications obtained by using the SIRC-CVS assay were compared with those obtained *in vivo*, 17 substances were accurately classified as negative by the SIRC-CVS assay, as shown in Table 2. There were ten false-positives and one false-negative. Eleven ingredients could not be tested, because a uniform suspension in culture medium could not be obtained. Nineteen substances that were deemed positive, and 11 which were poorly soluble or poorly suspensible in the culture medium of the SIRC-CVS assay, were tested in the LDM-MTT assay as the next step. All 11 substances could be applied to the LDM assay by using one of the four solvents described. When the classifications obtained by using the

LDM-MTT assay were compared with the *in vivo* data, 14 substances were truly negative, as shown in Table 3. There were no false-negatives and there were seven false-positives. Finally, 31 of 38 ingredients were classified correctly as non-irritating to the eye by using the tier system, as shown in Table 4. There were seven false-positives and one false-negative, which was phenethyl alcohol. The sensitivity, specificity, positive predictivity, negative predictivity and accuracy of the tier system were 90%, 82%, 56%, 97% and 83%, respectively.

Prediction of eye irritation at various test substance concentrations

Since it is desirable to predict the concentration at which an ingredient can be formulated into products without causing eye irritation, the LDM-MTT assay was assessed at various test substance concentrations. The concentrations of ingredients used were similar to those used in the previously-reported *in vivo* tests. As data were available at several concentrations for some of the 59 ingredients, the total number of *in vivo* data was 108, as

Table 4: Predicted irritancy according to an *in vitro* tier evaluation system comprising the SIRC-CVS assay and the LDM-MTT assay

		***In vitro* (classification by the tier evaluation system)**	
		+ve	**−ve**
***In vivo* (classification by Draize eye test at 10% conc.)** **Corneal damage or MAS over 15 was classified as positive**	**+ve**	**9** 2-Bromo-2-nitropropane-1,3-diol Benzalkonium chloride Cetrimonium chloride Chlorhexidine digluconate Chlorophene Dioctyl sodium sulphosuccinate Lauramide DEA Stearalkonium chloride TEA-Lauryl sulphate	**1** Phenethyl alcohol
	−ve	**7** Benzophenone-1 Benzophenone-2 Diazolidinyl urea PEG-40 stearate Phytantriol Sodium stearate Steareth-20	**31** Acetyl tributyl citrate Butylene glycol Carnauba wax Castor seed oil Cetyl alcohol Cetyl palmitate Decyl oleate Diethylhexyl adipate Diisopropyl adipate Ethylhexyl palmitate Ethylhexyl stearate Glyceryl stearate Hexylene glycol Isocetyl stearate Isopropyl myristate Isopropyl palmitate Oleyl alcohol PEG-2 stearate Propylene carbonate Safflower oil Sesame oil Sodium dehydroacetate Sorbitan oleate Sorbitan sesquioleate Sorbitan stearate Squalane Steareth-2 Stearyl alcohol Triacetin Triethylene glycol Zinc stearate

Table 5: Prediction of eye irritancy at various concentrations in the LDM-MTT assay

		In vitro (classification by LDM-MTT assay, with a viability of 50% as the cut-off point)	
		+ve	−ve
		42	**0**
	+ve	2-Bromo-2-nitropropane-1,3-diol (100, 20, 10, 5%) Benzalkonium chloride (2, 1, 0.5%) Benzophenone-1 (100%) Benzophenone-2 (100%) Butoxyethanol (100, 15%) Cetrimonium chloride (2.5, 1.2, 0.5%) Chlorhexidine digluconate (20, 2%) Chlorophene (100, 3%) Chloroxylenol (100, 30%) Dioctyl sodium sulphosuccinate (10%) Hexylene glycol (100%) Lauramide DEA (20, 10%) Methoxyisopropyl acetate (100%) Phenethyl alcohol (100, 15, 5%) Phenoxyethanol (100%) Phytantriol (100, 23%) Resorcinol (100%) Sodium naphthalenesulphonate (100%)	
***In vivo* (classification by Draize eye test)**		Stearalkonium chloride (25, 4, 2.5%) TEA-lauryl sulphate (20, 10, 5, 2.5, 1.25%) Triisopropanolamine (100%)	
		33	**33**
Corneal damage or MAS over 15 was classified as positive	−ve	2-Bromo-2-nitropropane-1,3-diol (2, 0.5%) Benzalkonium chloride (0.1, 0.01%) Benzethonium chloride (0.5%) Benzophenone-1 (16, 8, 4%) Benzophenone-2 (16, 8, 4%) Cetrimonium chloride (0.1%) Cetyl alcohol (100%) Cetyl palmitate (100%) Chlorhexidine digluconate (0.05%) Chlorophene (1, 0.3%) Diazolidinyl urea (30%) Diisopropyl adipate (100%) Dioctyl sodium sulphosuccinate (2, 0.5%) PEG-40 stearate (100%) Phytantriol (10, 3%) Propylene carbonate (100%) Sodium dehydroacetate (100%) Sodium lauroyl sarcosinate (5%) Sodium naphthalenesulphonate (2%) Sodium stearate (100%) Stearalkonium chloride (0.5%) Steareth-2 (60%) Steareth-20 (60%) Triacetin (100%)	Acetyl tributyl citrate (100%) Buthoxyethanol (5%) Butylene glycol (100, 10%) Carnauba wax (50%) Castor seed oil (100%) Decyl oleate (100%) Diethylhexyl adipate (100%) Ethylhexyl palmitate (100%) Ethylhexyl stearate (100%) Glyceryl stearate (100%) Hexylene glycol (25%) Isocetyl stearate (100%) Isopropyl myristate (100%) Isopropyl palmitate (100%) Oleyl alcohol (100%) PEG-2 stearate (100%) Phenethyl alcohol (0.3%) Phenoxyethanol (2.2%) Phenyl methyl pyrazolone (0.66%) Propylene carbonate (17.5, 10.5%) Safflower oil (100%) Sesame oil (100%) Sodium hexametaphosphate (0.2%) Sorbitan oleate (100%) Sorbitan sesquioleate (100, 30%) Sorbitan stearate (30%) Squalane (100%) Stearyl alcohol (100%) Triethylene glycol (100%) Zinc stearate (100%)

The LDM-MTT assay was performed at the concentration at which a reported in vivo *result was previously obtained. The concentrations of the substances at which they were classified as true-positive, true-negative, false-positive or false-negative, are shown in parentheses.*

shown in Table 1. When the classifications thus obtained were compared with the reported *in vivo* data, 33 compounds were correctly classified as negative by the LDM-MTT assay, as shown in Table 5, with no false-negatives and 33 false-positives. Based on these data for the LDM-MTT assay, the sensitivity, specificity, positive predictivity, negative predictivity and accuracy of the tier system were 100%, 50%, 56%, 100% and 69%, respectively.

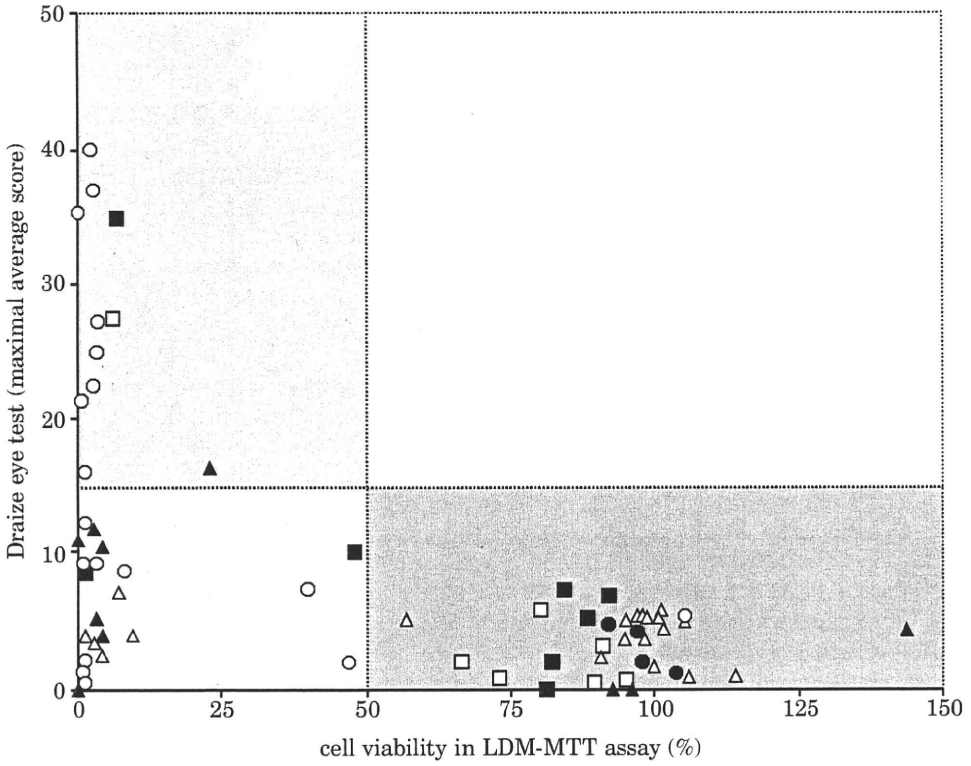
Relationship between viability in the LDM-MTT assay and the Draize eye test score

The prediction of the concentration at which an ingredient can be formulated into products without causing eye irritation was also examined with another 73 ingredients, for which MAS values in the Draize eye test had previously been obtained. The LDM-MTT assay was performed with similar concentrations of ingredients to those used in the corresponding *in vivo* tests. Thirty-nine substances whose treatment resulted in a viability of 50% or more in the LDM-MTT assay had an MAS of 15 or less in the Draize eye test, as shown in Figure 2. Eleven substances giving an MAS higher than 15 gave results which indicated a lower viability than 50% in the LDM-MTT assay. The sensitivity, specificity, positive predictivity, negative predictivity and accuracy of the tier system in this case were 100%, 60%, 31%, 100% and 66%, respectively.

Discussion

The tier evaluation system, comprising the SIRC-CVS assay and the LDM-MTT assay, for the identification of ingredients which are not ocular irritants, was designed on the basis of the results of

Figure 2: The relationship between the LDM-MTT assay and Draize eye test results for cosmetic ingredients



● = *UV absorbers (4);* ○ = *surfactants (19);* ■ = *macromolecules (10);* □ = *humectants (7);* △ = *oils (20);* ▲ = *medicants (13).*

= *true-positive (11);* = *false-positive (25);* = *true-negative (37);* = *false-negative (0).*

The classification in the Draize eye test was based on MAS 15 as the cut-off point. Classification in the LDM-MTT assay was based on a viability of 50% as the cut-off point.

Japanese validation studies (7). Factors taken into account in developing the tier evaluation system were as follows: the resources required to perform the tests (i.e. time, cost and manpower); the need for its wide applicability to a range of test substances; and, most importantly, the ability to predict eye irritancy through its use. The SIRC-CVS assay, which requires fewer resources, was conducted first. The second assay, the LDM-MTT assay, has the advantage that it is applicable to all types of substances, regardless of their solubility or form (liquid or powder). The LDM-MTT assay is also expected to be applicable to the prediction of the maximum concentration at which an ingredient can be formulated into products without causing eye irritation. It is important to note that differences in results between the two models can arise, not only from the differences in test conditions, but also from differences in the cell environment, including the effects of the culture medium. Therefore, the use of combinations of test models may be meaningful from the viewpoint of broadening their predictive ability. Further verification of the tier system was performed in the present study.

The 59 substances used for verification of the tier system (except for butylene glycol) were selected on the basis of the availability of *in vivo* eye irritation data in the Cosmetic Ingredient Review (CIR), and on their commercial availability. Relatively few substances met both criteria.

The SIRC-CVS assay could not be applied to 11 of 48 substances (Table 2), because those substances could not be suspended uniformly in the culture medium. However, these 11 substances could be tested in the LDM-MTT assay (Table 3). Thus, the limitations of SIRC-CVS method could be overcome by the subsequent use of the LDM-MTT assay. When eye irritancy at a concentration of 10% was predicted by using the tier system, there were good *in vitro–in vivo* correlations for 40 of 48 substances (Table 4). There was one false-negative (phenethyl alcohol), but seven false-positives. The tier system achieved high sensitivity and high negative predictivity. When eye irritancy at various concentrations of test compounds was predicted, the tier system again showed a similarly high sensitivity and negative predictivity (Table 5). On the other hand, the specificity and positive predictivity were not good. False positives seemed to appear, regardless of the class or type of substance, and could be at least partly a consequence of differences in the test concentrations used *in vivo* and in this sensitive *in vitro* test system. A similar tendency was seen in an additional study involving 73 other ingredients (Figure 2). Thus, the tier system is suitable for the identification of cosmetic ingredients which are not ocular irritants.

It is important that the number of false negatives should be small, from the viewpoint of consumer protection. Water-insoluble lower alcohols, such as phenethyl alcohol (a false-negative), should be checked by evaluation of the structure–activity relationship, before *in vitro* testing. Butanol and benzyl alcohol, which are also water-insoluble lower alcohols, were found to be false-negative substances in the previous Japanese validation study (6). In the case of false-positives, it is important to compare the IC50 values of potential false-positives with those of standard substances selected by taking into account existing experience in the market, the type of usage, physicochemical properties, etc., as shown in Figure 1.

In this study, the criterion for eye irritancy was taken as a reported appearance of corneal damage or a reported MAS of over 15 in the Draize eye test. The importance of the corneal damage criterion in the evaluation of cosmetic ingredients was discussed in the Japanese validation study. The criterion of MAS-over-15 for cosmetic ingredients was proposed by Ohno *et al.* (6), although a criterion of MAS-over-5 was also proposed, to provide a greater safety margin (5). When the *in vivo* data of the Japanese validation study were re-analysed from the viewpoint of recovery of ocular damage, eye irritation with an MAS value of 15 or less was shown to recover within 1 week of the application of the chemical, except for strong acids and strong alkalis. Thus, the level of MAS 15 without the appearance of corneal damage seemed acceptable as an index for eye irritation evaluation of cosmetic ingredients, though it may not be applicable to all ingredients — for example, some surfactants which are used in rinse-off products. Strong acid and alkaline character should be checked by the evaluation of physicochemical properties before *in vitro* testing.

The Global Harmonised System (GHS) is becoming increasingly important as an international standard of classification and labelling of chemicals. From the viewpoint of harmonisation of GHS standards and the standards for the safe use of cosmetic ingredients and medicated cosmetic ingredients in Japan, it should be noted that the *in vivo* value separating non-irritants and irritants in category 2B of the GHS is around MAS 15, on the basis of the *in vivo* data from the Japanese validation study and the GHS classification and modified MAS data reported by Van Goethem *et al.* (64). Therefore, we consider that classifications of positive for corneal damage or MAS-over-15 might correspond to GHS 1, 2A or 2B, while negative classifications might be equivalent to non-irritant in the *in vivo* classification of Tables 2–5. However, it is important to note that the GHS classification often cannot be precisely established from the available *in vivo* data. The standards for identifying non-irritants in the GHS might be adopted as acceptable standards of eye irritancy for ingredients of cosmetics and medicated cosmetics in

Japan, so it is relevant that the tier system, comprising the SIRC-CVS assay and the LDM-MTT assay, appears to be compatible with the GHS. The tier system is expected to further reduce the need to use animals for eye irritation testing.

Conclusions

This study has shown that the tier evaluation system, comprising SIRC monolayer cell cultures and a three-dimensional dermal model (LDM; MATREX), is a promising method for the identification of substances that are not ocular irritants. The tier evaluation system may be suitable for the evaluation of ingredients intended to be used in cosmetics and medicated cosmetics in Japan.

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A Comparative Evaluation of *In Vitro* Skin Sensitisation Tests: The Human Cell-line Activation Test (h-CLAT) *versus* the Local Lymph Node Assay (LLNA)

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Summary — We previously developed the human cell-line activation test (h-CLAT) *in vitro* skin sensitisation test, based on our reported finding that a 24-hour exposure of THP-1 cells (a human monocytic leukaemia cell line) to sensitisers is sufficient to induce the augmented expression of CD86 and CD54. The aim of this study is to confirm the predictive value of h-CLAT for skin sensitisation activity by employing a larger number of test chemicals. One hundred chemicals were selected, according to their categorisation in the local lymph node assay (LLNA), as being: extreme, strong, moderate and weak sensitisers, and non-sensitisers. The correlation of the h-CLAT results with the LLNA results was 84%. There were some false negatives (e.g. benzoyl peroxide, hexyl cinnamic aldehyde) and some false positives (e.g. 1-bromobutane, diethylphthalate). Eight out of the 9 false negatives (89%) were water-insoluble chemicals. The h-CLAT could positively predict not only extreme and strong sensitisers, but also moderate and weak sensitisers, though the detection rates of weak sensitisers and non-sensitisers were comparatively low. Some sensitisers enhanced both CD86 and CD54 levels, and some enhanced the level of only one of them. The use of the combination of CD86 and CD54 induction as a positive indicator, improved the accuracy of the test. In conclusion, the h-CLAT is expected to be a useful cell-based *in vitro* method for predicting skin sensitisation potential.

Key words: *human cell-line activation test, skin sensitisation, THP-1.*

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Introduction

Because of increasing social concerns about animal welfare, many alternative test methods have been proposed, especially for skin sensitisation testing (1). One of the most important approaches for developing alternative methods for skin sensitisation testing has been to measure phenotypic changes, such as the expression of CD86 or CD54 on the surface of dendritic cells (DCs) exposed to test agents (2, 3). However, the use of DCs is problematic, because the effects of chemicals on the surface phenotype of DCs were found to be dependent on the source of peripheral blood — that is, the effect varied from donor to donor (2, 4). Furthermore, peripheral blood as a source of DCs is not necessarily readily available. Therefore, we tested the human leukaemia cell line THP-1 as a substitute for DCs, and concluded that THP-1 cells, which show enhanced CD86 and/or CD54 expression when treated with sensitisers, can be used for *in vitro* skin sensitisation testing (5, 6). We named this test the human cell-line activation test (h-CLAT). In previous studies, we optimised the test conditions (7, 8), and we established that the h-CLAT can predict the sensitisation potential of a number of preservatives which are well-known sensitisers (9). Another group has also reported that THP-1 is a promising *in vitro* model for assays aimed at predicting the sensitisation potentials of chemicals, in that this cell-line is easy to handle and offers a number of practical advantages (10). Thus, the h-CLAT is expected to be a useful tool as a component of an *in vitro* test battery for predicting the sensitising properties of chemicals. Our inter-laboratory study in Japan revealed that the h-CLAT protocol is easy to transfer to other laboratories, and that the inter-laboratory reproducibility is basically good (11).

In the present study, in order to further confirm the predictive ability of the h-CLAT, we evaluated the skin sensitisation potential of 100 chemicals, by using the same protocol. High predictive ability is essential for a practical *in vitro* alternative test. Furthermore, analysis of the physicochemical properties and potency of false negatives should allow us to delineate the range of applicability and the limitations of this assay. The database of the local lymph node assay (LLNA) results consists of a large number of chemicals, and encompasses both chemical and biological diversity. Thus, it could help accelerate the development and valida-

tion of *in vitro* skin sensitisation tests. Therefore, we compared our proposed assay to the LLNA.

Materials and Methods

Laboratories

The h-CLAT was conducted independently by two laboratories. Almost all of the test chemicals were evaluated by both laboratories.

Cells and cell culture

THP-1 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (Invitrogen Corp., Carlsbad, CA, USA) with 10% (v/v) fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA), 0.05mM 2-mercaptoethanol and 1% (v/v) antibiotic–antimycotic mixture (Invitrogen Corp.).

The h-CLAT procedure

THP-1 cells were seeded at between 1×10^5 and 2×10^5 cells/ml, and pre-cultured for between 48 and 72 hours. After the incubation, the THP-1 cells were plated at 1×10^6 cells/ml in a 24-well plate (1ml per well) and treated for 24 hours with the test chemicals (2μl per well of each stock solution). The final concentration of DMSO in culture medium, when this was used as the vehicle, was less than 0.2% (v/v). Chemical-treated cells were washed twice with phosphate-buffered saline (PBS) solution containing 0.1% (w/v) bovine serum albumin (BSA), then treated with 0.01% (w/v) globulins, Cohn fractions II and III (Sigma-Aldrich, St Louis, MO, USA) for FcR blocking, for 10 minutes at 4°C. THP-1 cells have Fc-receptors on the cell surface, so it is advisable to block non-specific binding of monoclonal antibodies by pre-incubation of cells with globulins. Cell staining, with either Alexa Fluor®-conjugated anti-human CD86 antibody (clone Fun-1; BD-PharMingen San Diego, CA, USA; 1:7.3 dilution in FACS buffer), or fluorescein isothiocyanate (FITC)-conjugated anti-human CD54 antibody (clone 6.5B5; DAKO Glostrup, Denmark; 1:15.7 dilution in FACS buffer), was carried out at 4°C for 30 minutes. FITC-labelled mouse IgG1 (clone DAK-G01; DAKO) was used as an isotype control. The cells were washed twice with PBS containing 0.1% BSA, and the expression of cell surface antigens was analysed by flow cytometry. Dead cells were gated out by staining with propidium iodide (PI) at 0.625μg/ml. In total, 10,000 viable cells were analysed. When the cell viability was less than 50%, the relative fluorescence intensity (RFI) was not calculated, because of diffuse, non-specific labelling of cytoplasmic structures due to cell membrane destruction (12). The RFI was used as an indicator of CD86 and CD54 expression, and was calculated according to Equation 1 (see below).

Test chemicals and doses of application

The 100 test chemicals are listed in Table 1, with their Chemical Abstract Service (CAS) numbers. All the chemicals had been previously evaluated and classified with the LLNA (13). Seventy-two sensitisers were evaluated, including 8 extreme, 16 strong, 25 moderate, and 23 weak sensitisers, as classified by the LLNA; 28 non-sensitisers were also evaluated, of which one (sodium lauryl sulphate; SLS) was a false-positive in the LLNA. All the chemicals were purchased from Sigma-Aldrich, at the highest available purity. Either physiological saline or dimethylsulphoxide was used as the solvent. Application doses were determined from the results of the cytotoxicity tests conducted in each laboratory. Cytotoxicity was evaluated by flow cytometry after PI staining (the PI assay). From the PI assay data, eight doses based on the dose estimated to give 75% cell viability (CV75) were set, namely: $1.2 \times CV75$; $1 \times CV75$; $1/1.2 \times CV75$ (i.e. $0.8333 \times CV75$); $1/1.2^2 \times CV75$ (i.e. $0.6944 \times CV75$); $1/1.2^3 \times CV75$ (i.e. $0.5787 \times CV75$); $1/1.2^4 \times CV75$ (i.e. $0.4822 \times CV75$); $1/1.2^5 \times CV75$ (i.e. $0.4019 \times CV75$); and $1/1.2^6 \times CV75$ (i.e. $0.3349 \times CV75$). All the CV75 value doses of the test chemicals and the vehicles used in this study are shown in Table 1. We referred to an article recently published by ECVAM when selecting test chemicals (14). In particular, the chemical selection strategy described in the ECVAM article provided useful information. Because the common set of reference chemicals could be very useful in the method development phase, all of the 16 substances listed in the ECVAM article were adopted in the current study.

Data analysis

Tests were performed three times with each chemical. Two of three independent data at any dose

$$\text{RFI (\%)} = \frac{(\text{MFI of chemical-treated cells} - \text{MFI of chemical-treated isotype control cells})}{(\text{MFI of vehicle control cells} - \text{MFI of vehicle isotype control cells})} \times 100 \quad \text{[Equation 1]}$$

where MFI = (geometric) mean fluorescence intensity.

Table 1: Chemical information and h-CLAT data

Chemical	CAS No.	Molecular weight	Estimated Log $K_{o/w}$	Estimated water solubility (µg/ml)	LLNA		h-CLAT				
					Potency category	EC3 (%)	Result	CD86	CD54	CV75 (µg/ml)	Vehicle
Oxazolone	15646-46-5	217.2	1.51	1846	Extreme	0.003	P	+	–	166.6	DMSO
Diphenylcyclopropenone	886-38-4	206.3	3.25	67.58	Extreme	0.003	P	–	+	6.0	DMSO
MCI/MI (act. 1.5%)	Mixture	Variable	N.C.	1.49×10^5	Extreme	0.005	P	+	–	3.2	Saline
p-Benzoquinone	106-51-4	108.1	0.25	7.45×10^4	Extreme	0.0099	P	+	+	4.3	DMSO
1-Benzoylacetone	93-91-4	162.2	0.61	2.03×10^4	Extreme	0.04	P	–	+	92.8	DMSO
2,4-Dinitrochlorobenzene (DNCB)	97-00-7	202.6	2.27	242.5	Extreme	0.05	P	+	+	5.0	DMSO
4-Nitrobenzyl bromide	100-11-8	216.0	2.70	72.56	Extreme	0.05	P	+	+	3.6	DMSO
Potassium dichromate	7778-50-9	294.2	–3.59	1.00×10^6	Extreme	0.08	P	+	+	3.2	Saline
Glutaraldehyde (act. 50%)	111-30-8	100.1	–0.18	1.67×10^5	Strong	0.10	P	+	+	5.3	Saline
1,4-Dihydroquinone	123-31-9	110.1	1.03	1.30×10^5	Strong	0.11	P	+	–	5.0	Saline
1,4-Phenylenediamine*	106-50-3	108.1	–0.39	1.99×10^5	Strong	0.16	P	+	–	36.7	Saline
Phthalic anhydride	85-44-9	148.1	2.07	3326	Strong	0.16	N	–	–	400.0	DMSO
Maleic anhydride	108-31-6	98.1	1.62	4912	Strong	0.16	P	–	+	658.0	DMSO
Benzyl bromide	100-39-0	171.0	2.88	195.1	Strong	0.20	P	+	+	7.5	DMSO
Benzoyl peroxide*	94-36-0	242.2	3.43	28.97	Strong	0.30	N	–	–	41.0	DMSO
Lauryl gallate	1166-52-5	338.5	6.21	0.1371	Strong	0.30	P	+	+	8.2	DMSO
Propyl gallate	121-79-9	212.2	1.79	4176	Strong	0.32	P	–	+	125.0	DMSO
Cobalt chloride	7646-79-9	129.8	0.85	1.73×10^4	Strong	0.38	P	–	+	208.3	Saline
2-Aminophenol	95-55-6	109.1	0.60	3.24×10^4	Strong	0.40	P	+	–	6.0	DMSO
Chloramine-T	127-65-1	227.6	–0.50	8.37×10^4	Strong	0.40	P	+	+	314.7	Saline
2-Nitro-1,4-phenylenediamine	5307-14-2	153.1	0.55	2.59×10^4	Strong	0.50	P	–	+	490.7	DMSO
Formaldehyde (act. 37%)	50-00-0	30.0	0.35	5.70×10^4	Strong	0.61	P	+	+	5.8	Saline
Iodopropynyl butylcarbamate	55406-53-6	281.1	2.45	126.6	Strong	0.87	P	–	+	12.8	DMSO
Methyldibromoglutaronitrile	35691-65-7	265.9	1.63	424	Strong	0.90	P	+	+	9.9	DMSO
Isoeugenol*	97-54-1	164.2	2.65	165.9	Moderate	1.20	N	–	–	112.5	DMSO
1-Naphthol	90-15-3	144.2	2.69	1126	Moderate	1.30	P	+	+	57.2	DMSO
Glyoxal (act. 40%)	107-22-2	58.0	1.66	1.00×10^6	Moderate	1.40	P	–	+	396.0	Saline
2-Hydroxyethyl acrylate	818-61-1	116.1	0.25	5.07×10^5	Moderate	1.40	P	+	+	24.4	Saline
2-Mercaptobenzothiazole	149-30-4	167.2	2.86	543.4	Moderate	1.70	P	–	+	169.0	DMSO
Methylisothiazolinone (act. 9.7%)	2682-20-4	115.2	0.83	5.37×10^5	Moderate	1.90	P	+	+	24.7	Saline
3-Dimethylaminopropylamine	109-55-7	102.2	0.45	1.00×10^6	Moderate	2.20	P	+	+	276.7	Saline
Ethylenediamine	107-15-3	60.1	1.62	1.00×10^6	Moderate	2.20	P	+	–	271.7	Saline
1,2-Benzisothiazolin-3-one	2634-33-5	151.2	0.64	2.14×10^4	Moderate	2.30	P	–	+	1.83	DMSO
Methyl-2-nonynoate	111-80-8	168.2	3.10	142.5	Moderate	2.50	P	+	–	191.7	DMSO
Cinnamic aldehyde	104-55-2	132.2	1.82	2150	Moderate	3.00	P	+	+	28.0	DMSO

*CAS No. = Chemical Abstract Service number; N.C. = not calculated; DMSO = dimethyl sulphoxide; P = positive; N = negative; act. = actual concentration. *indicates that a substance is a pre/pro hapten.*

Table 1: continued

Chemical	CAS No.	Molecular weight	Estimated Log $K_{o/w}$	Estimated water solubility (μg/ml)	LLNA		h-CLAT				
					Potency category	EC3 (%)	Result	CD86	CD54	CV75 (μg/ml)	Vehicle
Phenylacetaldehyde	122-78-1	120.2	1.54	3026	Moderate	3.00	P	+	+	27.0	DMSO
3-Aminophenol	591-27-5	109.1	0.24	7.25×10^4	Moderate	3.20	P	–	+	243.2	DMSO
3-Propylidenephthalide	17369-59-4	174.2	2.03	1087	Moderate	3.70	P	+	+	112.9	DMSO
Benzylideneacetone	122-57-6	146.2	2.04	1345	Moderate	3.70	P	+	+	35.3	DMSO
α-Methylcinnamic aldehyde	101-39-3	146.2	2.37	752.8	Moderate	4.50	P	+	+	139.0	DMSO
Nickel sulphate hexahydrate	10101-97-0	262.8	2.04	1345	Moderate	4.80	P	+	+	150.0	Saline
Tetramethylthiuramdisulphide	137-26-8	154.8	0.17	1.01×10^5	Moderate	5.20	P	+	+	10.0	DMSO
3,4-Dihydrocoumarin	119-84-6	148.2	0.97	1.15×10^4	Moderate	5.60	P	–	+	810.0	DMSO
Resorcinol	108-46-3	110.1	1.03	8.57×10^4	Moderate	5.50	P	–	+	613.4	Saline
Diethylenetriamine	111-40-0	103.2	2.13	1.00×10^6	Moderate	5.80	N	–	–	1221.6	Saline
Diethyl maleate	141-05-9	172.1	2.20	800.8	Moderate	5.80	P	+	–	120.0	DMSO
2-Methoxy-4-methylphenol	93-51-6	138.2	1.88	2093	Moderate	5.80	P	+	+	280.0	DMSO
4-Chloroaniline	106-47-8	127.6	1.72	2572	Moderate	6.50	P	+	–	200.0	DMSO
Trimellitic anhydride	552-30-7	192.1	1.95	1036	Moderate	9.20	P	+	–	250.0	DMSO
1-Bromohexane	111-25-1	165.1	3.63	36.88	Weak	10.00	N	–	–	163.3	DMSO
Amyl cinnamic aldehyde	122-40-7	202.3	4.33	8.545	Weak	11.00	P	–	+	24.2	DMSO
Hexyl cinnamic aldehyde	101-86-0	216.3	4.82	2.75	Weak	11.00	N	–	–	37.0	DMSO
2,3-Butanedione	431-03-8	86.1	1.34	1.00×10^6	Weak	11.00	P	+	+	94.0	Saline
Citral	5392-40-5	152.2	3.45	84.71	Weak	13.00	P	+	+	24.0	DMSO
Eugenol*	97-53-0	164.2	2.73	754	Weak	13.00	P	+	+	143.2	DMSO
Abietic acid*	514-10-3	302.5	6.46	0.0896	Weak	15.00	N	–	–	89.6	DMSO
Oxalic acid	144-62-7	90.0	1.74	1.00×10^6	Weak	15.00	P	+	–	1000.0	DMSO
4-Allylanisole	140-67-0	148.2	3.47	84.55	Weak	18.00	P	–	+	186.0	DMSO
Lilial	80-54-6	204.3	4.36	7.859	Weak	19.00	P	–	+	42.2	DMSO
Phenyl benzoate	93-99-2	198.2	3.04	38.39	Weak	20.00	P	+	+	500.0	DMSO
Cinnamic alcohol*	104-54-1	134.2	1.84	6188	Weak	21.00	P	+	+	350.0	DMSO
Cyclamen aldehyde	103-95-7	190.3	3.91	22.59	Weak	22.00	N	–	–	49.8	DMSO
Benzocaine	60-09-3	197.2	3.19	20.46	Weak	22.00	P	+	–	545.5	DMSO
Imidazolidinyl urea	39236-46-9	388.3	8.28	1.00×10^6	Weak	24.00	P	+	+	40.1	Saline
Geraniol*	106-24-1	154.3	3.47	255.8	Weak	26.00	P	+	–	140.0	DMSO
Ethyleneglycol dimethacrylate	97-90-5	198.2	2.21	580.5	Weak	28.00	P	–	+	563.6	DMSO
Linalool	78-70-6	154.3	3.38	683.7	Weak	30.00	P	–	+	290.0	DMSO
Penicillin G	61-33-6	334.4	1.85	210.4	Weak	30.00	P	–	+	5000.0	DMSO
Butyl glycidyl ether	2426-08-6	130.2	1.08	2.66×10^4	Weak	31.00	N	–	–	185.3	DMSO
Hydroxycitronellal	107-75-5	172.3	2.11	3042	Weak	33.00	P	+	+	700.0	DMSO

*CAS No. = Chemical Abstract Service number; N.C. = not calculated; DMSO = dimethyl sulphoxide; P = positive; N = negative; act. = actual concentration. *indicates that a substance is a pre/pro hapten.*

Table 1: continued

Chemical	CAS No.	Molecular weight	Estimated Log $K_{o/w}$	Estimated water solubility (μg/ml)	LLNA		h-CLAT				
					Potency category	EC3 (%)	Result	CD86	CD54	CV75 (μg/ml)	Vehicle
Pyridine	110-86-1	79.1	0.80	7.30×10^5	Weak	72.00	P	+	–	4166.7	Saline
Aniline	62-53-3	93.1	1.08	2.08×10^4	Weak	89.00	P	+	+	930.0	DMSO
Acetanisole	100-06-1	150.2	1.75	2474	Non-sensitiser	N.C.	N	–	–	426.0	DMSO
Benzalkonium chloride	8001-54-5	340.0	2.93	22.47	Non-sensitiser	N.C.	N	–	–	3.0	Saline
Benzoic acid	65-85-0	122.1	1.87	2493	Non-sensitiser	N.C.	N	–	–	1000.0	DMSO
1-Bromobutane	109-65-9	137.0	2.65	386.2	Non-sensitiser	N.C.	P	+	+	500.0	DMSO
1-Butanol	71-36-3	74.1	0.84	7.67×10^4	Non-sensitiser	N.C.	N	–	–	1000.0	DMSO
Chlorobenzene	108-90-7	112.6	2.64	400.5	Non-sensitiser	N.C.	P	+	–	597.2	DMSO
Dextran	9004-54-0	Variable	N.C.	N.C.	Non-sensitiser	N.C.	N	–	–	5000.0	Saline
Diethyl phthalate	84-66-2	222.2	2.65	287.2	Non-sensitiser	N.C.	P	–	+	600.0	DMSO
Dimethyl formamide	68-12-2	73.1	–0.93	9.78×10^5	Non-sensitiser	N.C.	N	–	–	5000.0	Saline
Ethyl benzoylacetate	94-02-0	192.2	1.71	1212	Non-sensitiser	N.C.	N	–	–	571.1	DMSO
Ethyl vanillin	121-32-4	166.2	1.55	2867	Non-sensitiser	N.C.	N	–	–	569.5	DMSO
Glycerol	56-81-5	92.1	–1.65	1.00×10^6	Non-sensitiser	N.C.	N	–	–	5000.0	Saline
4-Hydroxybenzoic acid	99-96-7	138.1	1.39	1.45×10^4	Non-sensitiser	N.C.	N	–	–	> 1000	DMSO
2-Hydroxypropyl methacrylate	923-26-2	144.2	0.72	3.86×10^4	Non-sensitiser	N.C.	N	–	–	> 1000	DMSO
Isopropanol	67-63-0	60.1	0.28	4.02×10^5	Non-sensitiser	N.C.	N	–	–	> 5000	Saline
Lactic acid	50-21-5	90.1	–0.65	1.00×10^6	Non-sensitiser	N.C.	N	–	–	2800	Saline
6-Methylcoumarin	92-48-8	160.2	2.06	1189	Non-sensitiser	N.C.	N	–	–	276.9	DMSO
Methyl salicylate	119-36-8	152.2	2.60	1875	Non-sensitiser	N.C.	N	–	–	542.4	DMSO
Octanoic acid	124-07-2	144.2	3.03	495.9	Non-sensitiser	N.C.	P	–	+	359	DMSO
Propylene glycol	57-55-6	76.1	–0.78	8.11×10^5	Non-sensitiser	N.C.	N	–	–	> 1000	Saline
Propyl paraben	94-13-3	180.2	2.98	529.3	Non-sensitiser	N.C.	P	+	+	106.7	DMSO
Saccharin	81-07-2	183.2	0.45	789.2	Non-sensitiser	N.C.	N	–	–	> 1000	DMSO
Salicylic acid	69-72-7	138.1	2.24	3808	Non-sensitiser	N.C.	P	–	+	> 1000	DMSO
Streptomycin sulphate	3810-74-0	677.6	–11.83	1.00×10^6	Non-sensitiser	N.C.	N	–	–	> 1000	Saline
Tween-80	9005-65-6	1089.4	0.70	0.01999	Non-sensitiser	N.C.	N	–	–	> 5000	Saline
Vanillin	121-33-5	152.2	1.05	6875	Non-sensitiser	N.C.	N	–	–	650.0	DMSO
Zinc sulphate	7733-02-0	161.5	–0.07	7.75×10^4	Non-sensitiser	N.C.	P	–	+	670.8	Saline
Sodium lauryl sulphate	151-21-3	288.4	1.69	616.8	False-positive	N.C.	N	–	–	60.0	Saline

*CAS No. = Chemical Abstract Service number; N.C. = not calculated; DMSO = dimethyl sulphoxide; P = positive; N = negative; act. = actual concentration. *indicates that a substance is a pre/pro hapten.*

should exceed the positive criterion of 'CD86 > 150 or CD54 > 200', in order for the test chemical to be considered as 'positive'.

The calculation of Log $K_{o/w}$

The octanol–water partition coefficient (Log $K_{o/w}$) value was calculated with EPI suite™ (Environmental Protection Agency, Washington, DC, USA) for each chemical.

Results

Chemical information

One hundred chemicals, all of which had been previously tested in the LLNA for sensitisation potential, were evaluated with the h-CLAT (see Table 1). The test chemicals were selected according to the results of the LLNA, to cover the whole range of relative allergenic potencies, and included 72 sensitisers (8 extreme, 16 strong, 25 moderate, and 23 weak allergens, as classified in the LLNA) and 28 non-sensitisers. The potency category distribution was very similar to that of the historical data set of LLNA results (13), and the range of chemical diversity was wide. For example, the test chemicals included aldehydes, ketones, quinines and aromatic amines, and the Log $K_{o/w}$ values ranged from less than –3 to greater than 5. However, the majority of the sensitisers (67%) had Log $K_{o/w}$ values in the range of –1 to 3, which was reasonable because chemicals in this range are known to show good skin permeability (15). It should be noted that DMSO was used as a vehicle for about twice as many chemicals, as compared with saline.

All of the sensitisers listed in Table 1 had a molecular weight (MW) of less than 400Da, which was consistent with the generally-accepted view that chemicals with low MW tend to exhibit better skin penetration. The non-sensitisers in the dataset had Log $K_{o/w}$ and MW distributions similar to those of the sensitisers. Therefore, positivity or negativity could not be judged solely from the physicochemical properties of the test chemicals. In summary, the test chemicals employed in this study showed great diversity of physicochemical properties and sensitising potency.

The dose-finding assay (cytotoxicity test)

Because most sensitisers have a positive response within the range of 65% to 90% cell viability (16), we conducted cytotoxicity tests for all the test chemicals as dose-finding assays. The CV75 values (concentrations giving 75% cell viability) covered a wide range, from 3μg/ml (for benzalkonium chloride) to more than 5000μg/ml (for Tween-80), as evident in Table 1. In general, the cytotoxicity of non-sensitisers tended to be weaker than that of strong sensitisers, though there were some exceptions. This was not surprising, because chemicals with skin sensitising potency generally react readily with biomolecules, such as proteins (17). Some non-sensitisers hardly influenced cell function. However, penicillin G, which showed almost no cytotoxicity (CV75 > 5000μg/ml), was correctly found to be positive. On the other hand, some non-sensitisers showed strong cytotoxicity (e.g. benzalkonium chloride; CV75 = 3μg/ml). Therefore, it was impossible to estimate the sensitising potencies of chemicals from the results of the cytotoxicity assay.

The correlation between h-CLAT results and LLNA results

The h-CLAT data on CD86 or CD54 expression level changes, and the judgement of positivity or negativity for each chemical, are shown in Table 1. The results of the LLNA and the h-CLAT are compared in Table 2. The accuracy (proportion of 'correct' outcomes) of the h-CLAT with respect to the LLNA was 84% (84/100). The sensitivity (the proportion of all positive chemicals that are correctly classified as positive) was 88% (63/72), the specificity (the proportion of all negative chemicals that

Table 2: The correlation between LLNA and h-CLAT results

		h-CLAT	
		Positive (70 total)	**Negative (30 total)**
LLNA	Positive (72 total)	63	9
	Negative (28 total)	7	21

The grey shading indicates that the same results were obtained in both assays. The numbers indicated refer to the number of tested chemicals designated to each classification.

Table 3: Effectiveness of the battery evaluation of CD86 and CD54

	No. of chemicals
Number of sensitisers classified in the LLNA	72
Number of h-CLAT-positives out of these sensitisers	63/72
h-CLAT-positives in which CD86 expression was enhanced	14/63
h-CLAT-positives in which CD54 expression was enhanced	19/63
h-CLAT-positives in which both CD86 and CD54 expression was enhanced	30/63

are correctly classified as negative) was 75% (21/28), the positive predictivity (the proportion of correct positive responses among substances determined as positive) was 90% (63/70), and the negative predictivity (the proportion of correct negative responses among substances determined as negative) was 70% (21/30). Compared with the *in vivo* data, there are both false negatives and false positives. The number of chemicals classified in the h-CLAT as being false-negative was nine (isoeugenol, phthalic anhydride, etc.). On the other hand, the number of chemicals classified as false-positive was seven (1-bromobutane, diethylphthalate, etc.). Because the accuracy was 80% or more, based on the results for 100 chemicals, it was considered that the predictive ability of the h-CLAT was good. In particular, the positive predictivity was very good (90%), which means that, if a chemical is judged as being positive in the h-CLAT, the likelihood that it is a sensitiser is very high. Thus, the h-CLAT is expected to be a useful method for predicting the skin sensitisation potentials of chemicals.

With regard to CD86 and CD54 expression, the criterion *CD86 > 150 or CD54 > 200* for positivity was used in the h-CLAT, i.e. a chemical is judged to be positive when either the CD86 or CD54 level is over the specified value. As shown in Table 3, 63 of 72 sensitisers were found to be positive in this assay. Of the 63 positives, 30 enhanced both CD86 and CD54 expression, 14 enhanced only CD86 expression, and 19 enhanced only CD54 expression. It is interesting that the CD86/CD54 expression patterns are dependent on the sensitiser. Furthermore, the expression pattern did not appear to be related to sensitising potency, or to the physicochemical properties of the test chemical. The use of the combined criterion for changes in either CD86 or CD54 expression, improved the accuracy of the *in vitro* alternative with respect to the LLNA.

False negatives

There were nine false negatives among the 72 sensitisers in this study. As shown in Table 4, when solutions for the application to THP-1 cells were prepared, eight of the nine false negatives had to be dissolved in DMSO, i.e. they are water-insoluble chemicals. There was only one false negative (diethylenetriamine) among the 19 water-soluble sensitisers. It is well known that submerged culture systems such as the h-CLAT have limitations in the testing of water-insoluble chemicals. Some sensitisers (hexyl cinnamic aldehyde, abietic acid, benzoyl peroxide, etc.) might not have been evaluated correctly, due to their lack of solubility.

Next, we considered the sensitivity of the h-CLAT. Table 5 shows the differences in detection rate, according to potency category. The h-CLAT correctly evaluated all of the eight extreme sensitisers as positive, so the detection rate was 100%. The detection rates of strong sensitisers and moderate sensitisers were 87.5% and 91.7%, respectively. Therefore, the h-CLAT could predict the potency of most sensitisers with moderate or greater potential. However, there were five false negatives in the 'weak' rank group, and seven false positives in the non-sensitiser group. The detection

Table 4: The effects of solvent on positive predictivity

	Number of sensitisers	
	DMSO as solvent	Saline as solvent
True positives (63 total)	45	18
False negatives (9 total)	8	1
(Total 72)	53	19